APPENDIX A

DECLARATION

I, LEE, Ah-Ra of HANOL INTELLECTUAL PROPERTY & LAW of 19th Floor, City Air Tower 159-9 Samsung-dong, Kangnam-ku, Seoul 135-973, KOREA, do hereby solemnly declare that I translated Application No. 10-2004-003610 from Korean into English for the purpose of filing the aforementioned application in Europe. I further declare that the translation is to the best of my knowledge and belief a true and correct translation into the English language.

Date : March 30, **2010**

Signature Lee Ah-Ra

KOREAN INTELLECTUAL PROPERTY OFFICE

This is to certify that the following application annexed hereto is a true copy from the records of the Korean Intellectual Property Office.

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[English title of Invention] RAPID SCREENING METHOD OF SUITABLE TRANSLATIONAL FUSION PARTNERS FOR PRODUCING RECOMBINANT PROTEINS

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[Submitting Reason] We hereby file this application in accordance with the provision of Article 42 under the Patent Act.

Attorney SON, Min (Sign)

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ABSTRACT

[Abstract]

Disclosed are a method for rapid screening of suitable translational fusion partners (TFPs) capable of inducing expression or secretory production of non-producible proteins, which are difficult to produce in conventional recombinant production methods, from a variety of genetic sources, and protein secretion-inducing TFPs obtained using the method.

[Representative Figure]

FIG. 6

[Index]

translational fusion partners, yeast, non-producible protein, invertase

DESCRIPTION

[Title of the Invention]

RAPID SCREENING METHOD OF SUITABLE TRANSLATIONAL FUSION PARTNERS FOR PRODUCING RECOMBINANT PROTEINS

[Brief Description of the Drawings]

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 shows a process of deleting the invertase gene and a pop out process of a selectable marker;

FIG. 2 shows zymogram analysis for invertase activity (lanes 1, 2 and 3: wild-type *Saccharomyces cerevisiae* Y2805; and lanes 4, 5 and 6: invertase-deficient strain (*S. cerevisiae* Y2805 *Δinv2*);

FIG. 3 photographically shows the growth of yeast cells according to carbon sources (*INV2*: wild-type *S. cerevisiae* Y2805; and *Δinv2*: invertase-deficient strain (*S. cerevisiae* Y2805 *Δinv2*);

FIG. 4 shows the results of Southern blotting for the deletion of

the invertase gene (laens 1 and 2: *S. cerevisiae* Y2805 *ura3 INV2*; lanes 3 and 4: *S. cerevisiae* Y2805□*inv2U* (*URA3*□*inv2*); and lanes 5 and 6: *S. cerevisiae* Y2805□*inv2* (*ura3*□*inv2*);

FIG. 5 photographically shows the growth of yeast cells on glucose and sucrose media;

FIG. 6 shows a process of preparing pYHTS-F0, F1 and F2 plasmids and a process of preparing a yeast gene library;

FIG. 7 shows the results of SDS-PAGE and Western blotting for culture supernatants of yeast cells containing any of four translational fusion partners (lane 1: size marker; lane 2: interleukin-2; lane 3: culture supernatant of yeast cells containing pYIL-TFP1; lane 4: culture supernatant of yeast cells containing pYIL-TFP2; lane 5: culture

supernatant of yeast cells containing pYIL-TFP3; and lane 6: culture supernatant of yeast cells containing pYIL-TFP4);

FIG. 8 shows the results of glycosylation analysis by Endo-H digestion, wherein samples are analyzed on SDS-PAGE (lane 1 (-): culture supernatant of yeast cells containing pYIL-TFP1, not treated with Endo-H; lane 1 (+): culture supernatant of yeast cells containing pYIL-TFP1, treated with Endo-H; lane 2 (-): culture supernatant of yeast cells containing

pYIL-TFP3, not treated with Endo-H; lane 2 (+): culture supernatant of yeast cells containing pYIL-TFP3, treated with Endo-H; lane 3 (-): culture supernatant of yeast cells containing pYIL-TFP4, not treated with Endo-H; and lane 3 (+): culture supernatant of yeast cells containing pYIL-TFP4, treated with Endo-H);

FIG. 9 shows the results of SDS-PAGE of culture supernatants of yeast cells according to the presence or absence of a Kex2p possessing site (lane 1: pYIL-TFP1; lane 2: pYIL-KRTFP1; lane 3: pYIL-TFP3; lane 4:

pYIL-KRTFP3; lane 5: pYIL-TFP4; and lane 6: pYIL-KRTFP4);

FIG. 10 is a schematic presentation of plasmids from which the TFP1 gene has been partially deleted for the analysis of characteristics of TFP1;

FIG. 11 shows the results of SDS-PAGE for analyzing the ability of TFP1-derived translational fusion partners (TFP-1, 2, 3 and 4) to secrete interleukin-2 (lane M: size marker; lane S: interleukin-2; lane 1-1: culture supernatant of yeast cells containing pYIL-KRT1-1; lane 1-2: culture supernatant of yeast cells containing pYIL-KRT1-2; lane 1-3: culture

supernatant of yeast cells containing pYIL-KRT1-3; lane 1: culture supernatant of yeast cells containing pYIL-KRTFP1; and lane 1-4: culture supernatant of yeast cells containing pYIL-KRT1-4) ;

FIG. 12 shows the results of SDS-PAGE and Western blotting, displaying that translational fusion partners TFP1 induces the secretion of a non-producible protein human G-CSF (lane M: size marker; lane 1: culture supernatant of yeast cells containing pYIL-KRTFP1; lane 2: culture supernatant of yeast cells containing pYGCSF-TFP2; lane 3: culture supernatant of yeast cells containing pYGCSF-MFα).

[Detailed description of the Invention]

[Object of the Invention]

The present invention relates to a technique for rapid screening of suitable translational fusion partners (TFPs) capable of inducing expression

or secretory production of non-producible proteins, which are difficult to produce using conventional recombinant production methods, from a variety of genetic sources.

[Background and Technical Field]

There is a need to develop high-efficiency protein production systems using recombinant microorganisms to analyze human genome sequence data recently obtained through the Human Genome Project and functions of diverse proteins identified at genome units and to produce protein products important in human medical fields. When an expression system is selected to produce a recombinant protein derived from higher organisms such as humans, a variety of factors should be carefully considered, which include growth characteristics of host cells, protein expression levels, possibility of intracellular and extracellular expression, possibility of posttranslational modification, biological activity of expressed proteins. As representative microbial expression systems, *E. coli* and yeast systems are mainly used. *E. coli* is advantageous because many *E. coli*-based expression systems have been developed and *E. coli* expresses heterologous proteins in high levels. However, *E. coli* has the following drawbacks: the inability to perform posttranslational modification for recombinant production of proteins derived from higher eukaryotes, the difficulty in complete secretion of proteins into the culture medium, the lack of folding ability of proteins possessing many disulfide

bonds, and the expression of proteins in insoluble forms such as inclusion bodies (Makrides, Microbial Rev., 1996, 60, 512). In addition, since medically valuable disease-associated proteins among human proteins are mostly glycoproteins or membrane proteins, they need glycosylation and folding into a correct three-dimensional structure through disulfide bonds in order to achieve full activity. Thus, these proteins are impossible to produce in *E. coli* and essentially require eukaryotic expression systems such as yeasts.

Yeast *Saccharomyces cerevisiae* is a eukaryotic microorganism proven to be safe to the human body as a GRAS (Generally Recognized As Safe) organism. *S. cerevisiae* has many advantages including easy gene manipulation, various developed expression systems and easy large-scale culture. The advantages further include that *S. cerevisiae* functions to secrete higher cell-derived proteins such as human proteins into the extracellular space, and performs posttranslational modification of proteins, such as glycosylation. The extracellular secretion can be achieved through the artificial fusion of a target protein with a protein secretory signal, and during the secretion of a protein, protein folding or disulfide bond formation and glycosylation occur, thereby producing a fully biologically active recombinant protein. Also, since a biologically active protein can

be obtained directly from the culture medium, *S. cerevisiae*–based protein expression systems do not require cost-inefficient cell disruption or refolding so that they are very economical (Eckart and Bussineau, Curr. Opin. Biotechnol., 1996, 7, 525).

However, despite the many advantages of *S. cerevisiae* mentioned above, the problem of present techniques associated with systems for secreting human proteins using yeast *S. cerevisiae* involves non-uniform protein secretion yield ranging from no production to several grams/liter, depending on the human protein, leading to a great difference of more than several thousands in protein secretion yield, thus making it difficult to predict secretion yield. When a heterologous protein is secreted in several grams/liter, this protein production is considered to be cost-effective. In contrast, for the production of proteins expressed in low levels, especially highly valuable human therapeutic proteins, difficulties often occur in the expression and secretion of the proteins. To solve these problems, much research has been focused on secretory factors involved in protein secretion. For example, many studies have been carried out on chaperons, including a method of overexpressing a secretory factor, BiP (*KAR2*), which helps fold proteins newly synthesized in the endoplasmic reticulum(ER) (Robinson et al., Biotechnol. prog., 1996, 271, 10017), and a method of overexpressing *PDI* (protein disulfide isomerase) helping the formation of cysteine bonds (Robinson et al., Bio/Technology, 1994, 12, 381; Schultz et al., Ann. N. Y. Acad. Sci., 1994, 721, 148; Hayano et al., FEBS Lett., 1995, 377, 505). Also, another study has been performed to

improve secretion through preparation of a fusion partner inducing secretion and fusion with a well-secreted protein (Gouka et al., Appl. Microbiol. Biotechnol., 1997, 47, 1). To date, these methods have been considered to be very successful in improving the secretion of heterologous proteins. Molecular mechanisms of these fusion techniques have been poorly studied, but these fusion techniques have been experimentally proven to reduce limitations in translational or posttranslational steps, including facilitating protein translocation and helping protein folding.

Kjeldsen et al. (Protein Expr. Purif., 1997, 9, 331) enhanced the secretion of insulin by fusing insulin precursor with a synthetic leader prepared based on theoretical consideration in order to achieve effective secretion of insulin or insulin precursor. The synthetic leader has an N-glycosylation site and a BiP recognition site so that it extends the residence of the fusion protein in the ER, leading to correct folding of the insulin precursor. Also, the synthetic leader in which an additional glycosylation site is introduced remarkably increased the secretion of insulin in *Aspergillus niger* and *Saccharomyces cerevisiae* (Kjeldsen et al., Protein Expr. Purif., 1998, 14, 309). Similar results were obtained in *Aspergillus awamori* (Ward et al., Bio/Technology, 1989, 8, 435) and when cutinase is expressed in yeast (Sagt et al., Appl. Environ. Microbiol. 2000, 66, 4940). This high-yield secretion of recombinant proteins results from the introduction of glycosylation sites that increase the solubility of recombinant proteins in the ER and induce correct folding of the proteins.

Well-secreted proteins have been employed as fusion partners. For example, fusion expression with glucoamylase from *Aspergillus awamori* resulted in an increase in secretion yield of the following proteins: bovine prochymosin (Ward et al., Bio/Technology, 1989, 8, 435), porcine pancreatic phospholipase A2 (Roberts et al., Gene, 1992, 122, 155), human interleukin-6 (Contreras et al., Bio/Technology 1991, 9, 378; Broekhuijsen et al., J. Biotechnol., 1993, 31, 135), hen egg-white lysozyme (Jeenes et al., FEMS Microbiol Lett, 1993, 107, 267), and human lactoferrin (Ward et al., Bio/Technology, 1995, 13, 498). Increased secretion yield varied, depending on the protein, in a range of 5 to 1000 times. Also, the use of amino-terminal 24 amino acids of human interleukin-1□ as a fusion partner in yeast resulted in an increase in secretion yield of human growth hormone and granulocyte

colony-stimulating factor (G-CSF) (Lee et al., Biotechnol. Prog., 1999, 15, 884). Human interleukin-1□ is secreted without a particular secretory signal (Muesch et al., Trends Biochem. Sci., 1990, 15, 86), and its recombination production is very effective via secretion in yeast (Baldari et al., Protein Eng., 1987, 1, 433). Also, according to a recent report, a fusion partner originally retained in a protein is essential for correct folding of the protein (Takahashi et al., Appl Microbiol. Biotechnol., 2001, 55, 454). When the mature form of *Rhizopus oryzae* lipase (ROL) fused to the pre-pro-leader sequence of the mating factor alpha from *S. cerevisiae* was expressed in order to express ROL in *S. cerevisiae*, secretion of ROL was not observed. However, when ROL was synthesized together with the prosequence, ROL was properly secreted. These results demonstrate that the prosequence of ROL is essential for the folding of ROL itself.

[Technical Problem]

As described above, through much research, various seretory factors have been developed to induce the secretion of recombinant proteins. However, although the developed secretory factors are effective to increase the secretion level of particular proteins, they cannot be used as a general means for the secretory production of all proteins. Dorner et al. reported that overexpression of BiP in CHO cells rather reduces protein secretion (Dorner et al., EMBO J., 1992, 11, 1563), and decreased BiP expression increases protein secretion (Dorner et al., Mol. cell. Biol., 1988, 8, 4063). In yeast, overexpression of *KAR2* (BiP) did not enhance the

secretion of plant thaumatin (Harmsen et al., Appl. Microbiol. Biotechnol., 1996, 46, 365). Overexpression of BiP in Baculovirus resulted in an increase in levels of a soluble antibody in cell lysates but did not increase secretion yield of the antibody (Hsu et al., Protein Expr. Purif., 1994, 5, 595). When another secretory factor PDI as a foldase was overexpressed in *Aspergillus niger*, secretion of glucoamylase did not increase (Wang and Ward, Curr. Genet. 2000, 37, 57). Secretion improvement using a protein fusion partner was also reported to have a problem of increasing the secretion efficiency only of particular proteins.

As described above, much research has been focused on the effects of secretory factors, but secretory factors have different effects on secretion level depending on the types of proteins and thus cannot be applied to all proteins. Thus, there is a need for a technique of screening an optimal secretory factor specifically applicable to a target protein for maximal secretion of the target protein. In this regard, the present inventors developed a technique of rapidly screening an optimal secretory fusion partner from a genome unit according to types of recombinant proteins.

Accordingly, the present invention aims to provide a method capable of rapidly screening a suitable translational fusion partner (TFP) capable of strongly inducing production of a protein, which is unable to be produced at large scale and low cost due to its low expression levels in yeasts, from a variety of genetic sources including yeasts, and a translational fusion partner capable of stimulating the secretory production of a non-producible protein using the method.

[Best Mode]

In one aspect, the present invention relates to a method of screening, from a gene library, a translational fusion partner (TFP) that

induces extracellular secretion of a non-producible X-R fusion protein, which is prepared by linking a non-producible target protein gene (X) to a reporter gene (R) for automatic screening, through fusion of the X-R fusion product with other genes.

In one detailed aspect, the present invention relates to a method of screening a suitable translational fusion partner (TFP) for producing a non-producible protein, comprising:

(1) preparing an automatic screening vector including a fusion gene (X-R) in which a gene (X) encoding a non-producible target protein is linked in frame to a reporter gene (R) for automatic screening;

(2) linking a gene library including a TFP inducing secretion of the non-producible fusion protein (X-R) to the automatic screening vector

to create a TFP library;

(3) transforming cells having no activity of the reporter gene with the TFP library to detect the activity of a reporter protein; and

(4) isolating a gene from transformed cells exerting the activity of the reporter protein and analyzing properties of the TFP.

In a more preferred aspect, the present invention relates to a method of rapidly screening a suitable translational fusion partner (TFP) for producing a non-producible protein, comprising:

(1) preparing a yeast mutant strain deleted for its endogenous invertase gene INV2(I) to develop an automatic screening system using a yeast invertase as a reporter gene;

(2) preparing yeast high-throughput selection (HTS) vectors, pYHTS vectors (pYHTS-F0, pYHTS-F1 or pYHTS-F2) containing a gene (X-I) in which an invertase gene (I) is fused in frame to a non-producible protein gene (X) and which is controlled in expression under a yeast GAL10 promoter;

(3) preparing a translational fusion partner library from yeast

genes capable of secreting the fusion gene (X-I) of an invertase and a non-producible protein in the pYHTS vectors;

(4) transforming the library into the yeast mutant strain prepared at step (1) and performing automatic screening on a medium containing only sucrose as a carbon source;

(5) detecting a protein secreted into the medium by culturing yeast cells grown on the sucrose medium; and

(6) isolating genes from the yeast cells and analyzing properties of the translational fusion partner.

In one embodiment, when a non-producible protein (X), which is difficult to express in recombinant production, is fused to an invertase (I) and expressed in yeast cells, because the secretion of invertase secreted under normal conditions is inhibited by the fused poorly-secreted protein (X), the yeast cells do not grow or their growth is greatly delayed, due to poor expression levels of the fusion protein on a medium containing only sucrose as a carbon source. However, when an effective translational fusion partner capable of inducing the expression and secretion of X-I is introduced, cells rapidly grow on a sucrose medium. Based on this principle, when the X-I fusion protein of a non-producible protein and an invertase is additionally fused to a translational fusion partner library obtained from a variety of origins in the form of TFP-X-I or X-I-TFP, introduced into yeast cells and expressed therein, cells rapidly growing on the sucrose medium are selected, thereby allowing rapid screening of a TFP most suitable for the non-producible protein from a variety of libraries.

To be more specific, the present inventors prepared an invertase-deficient yeast mutant and found that invertase can be used as a marker for

automatic screening through the expression of a protein fused to the invertase in a yeast strain deleted for its invertase gene. Then, the present inventors prepared vectors for the automatic screening of a translational fusion partner, pYHTS-F0, F1 and F2, using a non-producible protein, human interleukin-2, linking yeast-derived cleaved chromosomal DNA to the vectors to generate a translational fusion partner library, and found, from the TFP library, TFP proteins suitable for the poorly secreted protein human interleukin-2, TFP1, TFP2, TFP3 and TFP4.

The term "translational fusion partner (TFP)", as used herein, refers to a gene that is fused to a gene encoding a non-producible protein and induces the secretory production of the non-producible protein. Also, the "translational fusion partner protein" means a protein having an amino acid sequence encoded by the aforementioned TFP gene. To be more specific, TFP1, TFP2, TFP3 and TFP4 can be enumerated.

Yeast cells need an invertase enzyme encoded by a yeast *INV2* gene using only sucrose as a carbon source. As used herein, the term automatic screening system using invertase means a system for selecting a yeast strain growing on a sucrose medium according to the expression of an *INV2* gene introduced into a vector while the yeast strain is deleted for its chromosomal *INV2* gene.

The reporter gene for automatic screening according to the present invention is selected from, but is not limited to, a gene group encoding proteins capable of being extracellularly secreted, including invertase, sucrase, cellulase, xylanase, maltase, amylase, glucoamylase and galactosidase.

The term non-producible protein, as used herein, refers to a protein that is difficult to express in host cells, such as *E. coli* or yeasts, due to its native properties with respect to recombinant production of proteins from humans or various organisms. In particular, with respect to the objects of the present invention, a non-producible protein is a protein that is difficult to express in host cells such as yeasts in recombinan`t production. The screening method of the present invention and the translational fusion partner obtained using the screening method are used for recombinant production of proteins that cannot be recombinantly produced in both prokaryotic cells such as *E. coli* and eukaryotic cells such as yeasts, as well as a plurality of proteins that can be recombinantly produced in prokaryotic cells such as *E. coli* but are cost-ineffective due to their low yield in eukaryotic cells such as yeasts.

Translational fusion partners TFP1, TFP2, TFP3 and TFP4 and derivatives thereof, which are obtained in the present invention, may be applied to a variety of proteins produced at commercial large scale. These proteins include, but are not limited to, cytokines (e.g., interleukin), serum proteins (e.g., coagulation factors including Factors VII, VIII and IX), immunoglobulins, cytokine receptors, lactoferrin, interferons (e.g., interferon-, - and -), colony stimulating factors (e.g., GM-CSF, G-CSF), phospholipase A2-activating protein (PLAP), insulin, tumor necrosis factor (TNF), growth factors (e.g., tissue growth factors and epithelial growth factors, such as TGF or TGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF)), hormones (e.g., follicle stimulating hormone, thyroid stimulating hormone, antidiuretic hormone, pigmenary hormone and parathyroid hormone, luteinizing hormone-releasing hormone and derivatives thereof), calcitonin, calcitonin gene related peptide (CGPR), enkephalin, somatomedin, erythropoietin, hypothalamic releasing factor, prolactin, chorionic gonadotropin, tissue plasminogen activator, growth hormone releasing peptide (GHPR), thymic humoral factor (THF), and anticancer and antibiotic peptides. Also, these proteins may include enzymes, which are exemplified by carbohydrate-specific enzymes, proteolytic enzymes, lipases, oxidoreductases, transferases, hydrolases, lyases, isomerases and

ligases. Concrete examples of enzymes include, but are not limited to, asparaginase, arginase, arginine deaminase, peroxide dismutase, endotoxinase, catalase, chymotrypsin, uricase, adenosine diphosphatase, tyrosinase, and bilirubin oxidase. Examples of the carbohydrate-specific enzymes include glucose oxidase, glucodase, galactosidase, glucocerebrosidase and glucuronidase.

Non-producible protein genes are genes that encode the aforementioned proteins having human medical or industrial importance, the recombinant production of which is required, and are isolated or chemically synthesized from genes derived from a variety of plants, animals and microorganisms including humans, or cDNA.

The automatic screening vector of the present invention includes a promoter gene, a gene encoding a target protein, which is deleted for translation initiation and termination codons, and a reporter gene fused in frame to the gene encoding the target protein. The promoter gene is preferably selected from the group consisting of *GAPDH, PGK, ADH, PHO5, GAL1* and *GAL10*. The gene library including a translational fusion partner for inducing the secretion of a non-producible fusion protein may be selected or chemically synthesized from a variety of origins, for example, animals, plants and microorganisms, including yeasts or humans. Preferred is a gene library from yeasts. The gene library may be in the form of genomic (chromosomal) DNA or cDNA.

In the automatic screening method of the present invention, the host cells to be transformed include, but are not limited to, yeasts, such as *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Yarrowia* and *Saccharomyces* species, fungi, such as *Aspergillus*, *Penicillium*, *Rhizopus* and *Trichoderma* species, and bacteria, such as *Escherichia* and *Bacillus* species.

The rapid screening method of a suitable TFP for producing a

non-producible protein according to the present invention is preferably used for producing non-producible proteins that are not expressed or are expressed in low levels. Also, the present method may be used for screening a TFP capable of increasing expression levels of a low-level expressed protein. As in one embodiment of the present invention, when the invertase enzyme is used as a reporter, cells are selected according to their growth rates on the sucrose medium, thereby allowing discrimination of more effective TFPs.

In another aspect, the present invention relates to vectors, pYHTS-F0, F1 and F2, for rapidly screening a suitable fusion partner for stimulating the secretory production of a non-producible protein

interleukin-2. These screening vectors include a fusion gene of the non-producible protein human interleukin-2 and invertase, and contain a *BamHI* recognition site having three different reading frames at an amino terminal end of the interleukin-2 gene.

In an embodiment of the present invention, in order to rapidly screen suitable fusion partners stimulating the secretory production of human interleukin-2 in yeast cells, yeast chromosomal DNA is randomly cleaved and inserted into the three screening vectors (pYHTS-F0, F1 and F2). A yeast strain lacking invertase is transformed with the resulting screening vectors, and colonies growing on a sucrose medium are selected to identify suitable fusion partners capable of secreting the fusion protein of

non-producible interleukin-2 and invertase into the culture medium.

Human interleukin-2, which is a highly hydrophobic protein, is difficult to express in yeast cells because the recombinant protein expressed at large scale by a strong promoter is not folded rapidly into an active form in the ER but forms aggregates that may block the function of the ER. Thus, when fused to interleukin-2, invertase is also not secreted, and yeast cells cannot grow on a sucrose medium. Translational fusion partners capable of effectively secreting this fusion protein may be identified by inserting a yeast genomic library upstream of the interleukin-2 gene, transforming the library into a yeast strain and selecting transformants growing on a sucrose medium.

In an embodiment, in order to obtain fusion partners inducing secretion of the non-producible protein interleukin-2, the present inventors isolated genes from transformants growing on a sucrose medium,

re-transformed the genes into *E. coli*, and recovered four different plasmids (pYHTS-TFP1, TFP2, TFP3 and TFP4). Four different translational fusion partner genes carried in the plasmids, TFP1 (SEQ ID NO. 2), TFP2 (SEQ ID NO. 4), TFP3 (SEQ ID NO. 6) and TFP4 (SEQ ID NO. 8), were obtained, and corresponding amino acid sequences are represented by SEQ ID NOS. 1, 3, 5 and 7, respectively.

The invertase gene was deleted in the obtained vectors pYHTS-TFP1, TFP2, TFP3 and TFP4, and a translation termination codon was inserted into the interleukin-2 gene, thus generating pYIL-TFP1, TFP2, TFP3 and TFP4. Since these vectors secrete interleukin-2 in the form of being fused to a translational fusion partner, a recognition site for Kex2p proteinase is inserted to allow automatic removal of the translational fusion

partner, thus generating pYIL-KRTFP1, KRTFP2, KRTFP3 and KRTFP4. Also, human granulocyte colony stimulating factor (G-CSF) is fused to the translational fusion partners TFP1 to TFP4, thus generating vectors pYGCSF-TFP1 to pYGCSF-TFP4, respectively. These vectors demonstrated that the TFPs are effective in secretory production of proteins other than human interleukin-2.

Thus, in a further aspect, the present invention relates to a translational fusion partner TFP1 protein represented by SEQ ID NO. 1 or an analogue thereof. Also, the present invention relates to a gene encoding a translational fusion partner TFP1 protein represented by SEQ ID NO. 1 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP1 protein having an amino acid sequence represented by SEQ ID NO. 1 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence encoding a translational fusion partner TFP1 protein represented by SEQ ID NO. 1, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95%

or higher homology. Preferably, the gene is a gene of SEQ ID NO. 2. Further, the present invention relates to a recombinant vector comprising the gene. Preferably, the gene carried in the recombinant vector is a gene of SEQ ID NO. 2. Examples of the recombinant vector include pYIL-TFP1, pYIL-KRTFP1, pYGCSF-TFP1, pYGCSF-KRTFP1 and pGAP-TFP1-GCSF. Still further, the present invention relates to a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRTFP1 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number DH5□/pYIL-KRTFP1(KCTC 10544BP).

In still another aspect, the present invention relates to a translational fusion partner TFP2 protein represented by SEQ ID NO. 3 or an analogue thereof. Also, the present invention relates to a gene encoding a translational fusion partner TFP2 protein represented by SEQ ID NO. 3 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP2 protein having an amino acid sequence represented by SEQ ID NO. 3 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence encoding a translational fusion partner TFP2 protein represented by SEQ ID NO. 3, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95%

or higher homology. Preferably, the gene is a gene of SEQ ID NO. 4. Further, the present invention relates to a recombinant vector comprising the gene. Preferably, the gene carried in the recombinant vector is a gene of SEQ ID NO. 4. Examples of the recombinant vector include pYIL-TFP2, pYIL-KRTFP2, pYGCSF-TFP2 and pYGCSF-KRTFP2. Still further, the present invention relates to a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRTFP2 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number as DH5□/pYIL-KRTFP2(KCTC 10545BP).

In still another aspect, the present invention relates to a translational fusion partner TFP3 protein represented by SEQ ID NO. 5 or an analogue thereof. Also, the present invention relates to a gene encoding a translational fusion partner TFP3 protein represented by SEQ ID NO. 5 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP3 protein having an amino acid sequence represented by SEQ ID NO. 5 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence encoding a translational fusion partner TFP3 protein represented by SEQ ID NO. 5, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95%

or higher homology. Preferably, the gene is a gene of SEQ ID NO. 6. Further, the present invention relates to a recombinant vector comprising the gene. Preferably, the gene carried in the recombinant vector is a gene of SEQ ID NO. 6. Examples of the recombinant vector include pYIL-TFP3, pYIL-KRTFP3, pYGCSF-TFP3, pYGCSF-KRTFP3 and pYGT3-CalB14. Still further, the present invention relates to a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRTFP3 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number DH5□/pYIL-KRTFP3(KCTC 10546BP).

In still another aspect, the present invention relates to a translational fusion partner TFP4 protein represented by SEQ ID NO. 7 or an analogue thereof. Also, the present invention relates to a gene encoding a translational fusion partner TFP4 protein represented by SEQ ID NO. 7 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP4 protein having an amino acid sequence represented by SEQ ID NO. 7 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence encoding a translational fusion partner TFP4 protein represented by SEQ ID NO. 7, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95%

or higher homology. Preferably, the gene is a gene of SEQ ID NO. 8. Further, the present invention relates to a recombinant vector comprising the gene. Preferably, the gene carried in the recombinant vector is a gene of SEQ ID NO. 8. Examples of the recombinant vector include pYIL-TFP4, pYIL-KRTFP4, pYGCSF-TFP4 and pYGCSF-KRTFP4. Still further, the present invention relates to a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRTFP4 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number DH5□/pYIL-KRTFP4(KCTC 10547BP).

The term "analogue", as used for a translational fusion partner protein or gene herein, means a functional equivalent that exerts the activity of the translational fusion partner by inducing secretory production of a non-producible protein when a translational fusion partner gene is fused to a gene encoding the non-producible protein. In the case of the TFP protein,

the analogue may include, for example, substitutions between amino acids having the same properties (e.g., replacement of a hydrophobic amino acid with another hydrophobic amino acid, replacement of a hydrophilic amino acid with another hydrophilic amino acid, replacement of a basic amino acid with another basic amino acid, replacement of an acidic amino acid with another acidic amino acid), deletions and insertions of amino acids, or combinations thereof.

The term "fragment" used for the translational fusion partner proteins or genes of the present invention means the translational fusion partner genes or proteins coded by these genes which may not affect or may stimulate the secretion of a non-producible protein even if a portion of the whole sequence of the translational fusion partner genes identified using a genomic library or a cDNA library is deleted.

The term homologous, as used for a translational fusion partner protein or gene herein, is intended to express similarity to the wild-type amino acid sequence and the wild-type nucleotide sequence. In case of the protein, homologous includes an amino acid sequence preferably 75% or higher, more preferably 85% or higher, even more preferably 90% or higher and most preferably 95% or higher identical to an amino acid sequence of the TFP protein of the present invention. Typically, protein homologues may include an active site identical to a target protein. In the case of the gene, homologous includes a DNA sequence preferably 75% or higher, more preferably 85% or higher, even more preferably 90% or higher and most preferably 95% or higher identical to a DNA sequence encoding the TFP protein of the present invention. The homology evaluation may be done with the naked eye or using a commercially available program. Using a commercially available computer program, the homology between two or more sequences may be expressed as a percentage (%), and the homology (%) between adjacent sequences may be evaluated.

The translational fusion partners identified according to the present invention for the secretory production of non-producible proteins are used in the form of being fused to a gene encoding a non-producible

protein and is inserted into a vector for the secretory production of the non-producible protein. The term vector, as used herein, refers to a DNA construct that contains a DNA sequence operably linked to regulatory sequences capable of controlling the expression of a protein in a suitable host and sequences introduced for facilitating other genetic manipulation or optimizing the expression of the protein. Such regulatory sequences include a promoter for transcription control, an operator selectively added for transcription control, a suitable mRNA ribosome binding site and sequences controlling termination of transcription/translation. Such a vector for insertion of an exogenous gene may be a plasmid, a virus, a cosmid, or the like. The vector includes cloning vectors and expression vectors. The cloning vector is a replicable plasmid into which exogenous DNA is inserted, and delivers exogenous DNA into host cells transformed therewith. The expression vector typically means a carrier into which a fragment of exogenous DNA, generally a fragment of double-stranded DNA, is inserted. Exogenous DNA refers to heterogeneous DNA that does not naturally occur in host cells. The expression vector is able to replicate independently of host chromosomal DNA in host cells so that inserted exogenous DNA may be produced. As generally known in the art, in order to increase the expression level of a transfected gene in a host cell, the gene should be operably linked to transcription and translation regulatory sequences functional in a host cell selected as an expression system.

The term transformation, as used herein with respect to transformation using a recombinant vector containing a translational fusion partner, means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration. Host cells useful for the transformation according to the present invention may be prokaryotic or eukaryotic. In addition, host cells having high transformation efficiency of foreign DNA and having high expression levels of an introduced DNA may be typically used. Examples of host cells include prokaryotic and eukaryotic cells such as *Escherichia* sp., *Pseudomonas* sp., *Bacillus* sp., *Steptomyces* sp., fungi and yeast, insect cells such as *Spodoptera frugiperda* (Sf9), and animal cells such as CHO, COS 1, COS 7, BSC 1, BSC 40 and BMT 10. *Escherichia coli* may be

preferably used.

The present inventors investigated the effect of deletion analogue fragments of the translational fusion partners TFP1, TFP2, TFP3 and TFP4 on the secretion of a non-producible protein. A vector carrying TFP1 that is deleted in a serine/alanine-rich sequence, an N-glycosylation site or both did not secrete a non-producible protein. In contrast, a vector (pYIL-KRT1-4)carrying TFP1 that has been deleted in the 5-UTR (5-untranslated region) increased expression levels of the non-producible protein over three times. Also, when the 3- end was additionally deleted (pYIL-KRT1-3), the secretion of the non-producible protein was induced. Thus, deletion analogues of the translational fusion partners of the present invention in a 5-UTRand partially deleted TFPs in 3-end are included in the scope of the present invention, as long as they do not negatively affect the secretion of a non-producible protein.

In a detailed aspect, the present invention provides a translational fusion partner TFP1-3 protein represented by SEQ ID NO. 9 or an analogue thereof. Also, the present invention provides a gene encoding a translational fusion partner TFP1-3 protein represented by SEQ ID NO. 9 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP1-3 protein having an amino acid sequence represented by SEQ ID NO. 9 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more

preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence encoding a translational fusion partner TFP1-3 protein represented by SEQ ID NO. 9, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Further, the present invention provides a recombinant vector comprising the gene. Preferably, the gene carried in the recombinant vector is a gene encoding a translational fusion partner TFP1-3 represented by SEQ ID NO. 9. An illustrative example of the recombinant vector is pYIL-KRT1-3. Still further, the present invention relates to a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRT1-3 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number DH5□/pYIL-KRT1-3(KCTC 10548BP).

In another detailed aspect, the present invention relates to a

translational fusion partner TFP1-4 protein encoded by a gene represented by SEQ ID NO. 10 or an analogue thereof. Also, the present invention relates to a gene encoding a translational fusion partner TFP1-4 and represented by SEQ ID NO. 10 or an analogue thereof. The scope of the present invention includes a translational fusion partner TFP1-4 protein having an amino acid sequence encoded by a gene represented by SEQ ID NO. 10 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. Also, the scope of the present invention includes a gene having a DNA sequence represented by SEQ ID NO. 10, or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. The present invention provides a recombinant vector comprising a gene encoding a translational fusion partner TFP1-4 and represented by SEQ ID NO. 10 or an analogue thereof. Also, the present invention provides a recombinant vector comprising a gene having a DNA

sequence encoding a translational fusion partner TFP1-4 and represented by SEQ ID NO. 10 or a DNA sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology. An illustrative example of the recombinant vector is pYIL-KRT1-4. Further, the present invention provides a cell transformed with the recombinant vector. *Escherichia coli* transformed with pYIL-KRT1-4 was deposited at KCTC (Korean Collection for Type Cultures) on November 11, 2003, and assigned accession number DH5□/pYIL-KRT1-4(KCTC 10549BP).

The method for the recombinant production of a non-producible protein comprises preparing an expression vector into which a coding gene of the non-producible protein, fused to a gene encoding the TFP protein, is inserted, and culturing a transformant transformed with the recombinant expression vector. In detail, the present invention relates to a method of

recombinantly producing a non-producible protein using a protein having an amino acid sequence represented by SEQ ID NO. 1, 3, 5, 7, or 9 or an amino acid sequence homologous thereto, having preferably 75%, more preferably 85%, even more preferably 90% and most preferably 95% or higher homology, or using a protein having an amino acid sequence encoded by a gene represented by SEQ ID NO. 10 or an amino acid sequence homologous thereto, having preferably 75% or higher, more preferably 85% or higher, even more preferably 90% or higher and most preferably 95% or higher homology. Preferably, the protein represented by SEQ ID NO. 1 is encoded by a gene represented by SEQ ID NO. 2, the protein represented by SEQ ID NO. 3 is encoded by a gene represented by SEQ ID NO. 4, the protein represented by SEQ ID NO. 5 is encoded by a gene represented by SEQ ID NO. 6, and the protein represented by SEQ ID NO. 7 is encoded by a gene represented by SEQ ID NO. 8. Preferably, the non-producible protein is human interleukin 2 or human G-CSF.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be constructed as the limit of the present invention.

EXAMPLE 1: Preparation of invertase-deficient yeast mutant

For rapid screening of translational fusion partners of non-producible proteins, an automatic screening system was established through the evaluation of cell growth in a sucrose medium using yeast

invertase as a reporter.

A yeast strain deficient for invertase activity was required to use the invertase gene contained in a vector as a reporter gene upon screening after transformation. Thus, the *INV2* gene was deleted in yeast chromosomal DNA. In order to prepare a cassette for inducing gene deletion, a pRB58 plasmid (Carlson et al., Cell, 1982, 20, 145) was digested with *Eco*RI and *Xho*I, and an *INV2* coding gene was recovered and introduced into *Eco*RI/*Xho*I sites of pBluescript KS+ (Stratagene, USA), thus generating pBI□BX. As shown in FIG. 1, an *URA3* gene having a repeat sequence of 190 bp (Tc190) at both its ends was inserted into

*Hin*dIII-*Xba*I sites of the *INV2* gene contained in the pBI□BX, thus

generating pBIU. The pBIU was digested with both *Eco*RI and *Xho*I, and was transformed into *Saccharomyces cerevisiae* Y2805Δ*gal1* (*Mat a ura3 INV2 pep4::HIS3 gal1 can1*) strain (SK Rhee, Korea Research Institute of Bioscience and Biotechnology). The transformant, Y2805Δ*gal1*Δ*inv2U* (*Mat a inv2::URA3 pep4::HIS3 gal1 can1)*, was selected in a selection medium lacking uracil.

The selected transformed cells were evaluated to determine whether they completely lost invertase activity. Single colonies were cultured in two media containing glucose and sucrose, respectively, as the sole carbon source. As a result, the colonies grew normally in the glucose medium, but grew very slowly in the sucrose medium compared to a control. In order to investigate the amount of invertase secreted into the culture medium, *INV2*+ strain and Δ*inv2* strain were cultured. Proteins

contained in the culture supernatants were separated on SDS-PAGE, and

the gel was incubated in a sucrose solution for 30 min and subjected to

zymogram analysis using a dye, TTC (2,3,5-triphenyl-tetrazolium chloride). As shown in FIG. 2, the Δ*inv2* strain was found to lose most of its invertase activity. However, the mutant strain had a problem of growing even at very slow rates in the sucrose medium. This is believed to be because cells partially grow by gluconeogenesis through the function of mitochondria. Thus, to solve this problem, antimycin A, an inhibitor of mitochondrial electron transport, was added to the medium to block cell growth. As a result, the growth of the mutant strain was completely inhibited in the presence of antimycin A (FIG. 3).

In order to re-transform the selected strain, Y2805Δ*gal1*Δ*inv2U* (*Mat a inv2::URA3 pep4::HIS3 gal1 can1)*, with a *URA3* vector containing a TFP library, it was necessary to remove the *URA3* gene used for the deletion of the *INV2* gene. To do this, cells were cultured in a medium containing 5-fluoroorotic acid (5-FOA) and selected for loss of the *URA3* gene, thus obtaining a *URA3* pop-out deletion strain, Y2805Δ*gal1*Δ*inv2*(*Mat a ura3 inv2::Tc190 pep4::HIS3 gal1 can1)* (FIG. 1). Southern blotting was carried out to confirm the deletion of the *INV2* gene on chromosome, as expected, and the pop□out of the *URA3* gene (FIG. 4). When chromosomal DNA from *S. cerevisiae* Y2805 was treated

with *Eco*RI and analyzed by Southern blotting using an *INV2* gene as a probe, a fragment of about 4.3 kb was detected. This size increased to about 5.0 kb when a *URA3* gene was inserted (Y2805Δ*gal1*Δ*inv2U*), and decreased to about 3.7 kb when the *URA3* gene was popped-out (Y2805Δ*gal1*Δ*inv2*). As shown in FIG. 4, as expected, the *INV2* gene was obviously deleted, and the *URA3* gene was lost (pop-out).

EXAMPLE 2: Identification of automatic screening system through fusion with invertase

The invertase gene-deficient strain was evaluated for the possibility of being automatically screened in a sucrose medium through the expression of a protein fused to invertase, using a human protein expressed in high levels in yeast, human serum albumin (HSA), and a non-producible protein, human interleukin-2 (IL-2).

First, a pGHSA-INV2 vector in which albumin is fused to invertase was prepared as follows. In order to insert a *Sfi*I recognition sequence into both ends of the HSA gene, PCR was carried out using a sense primer and an anti-sense primer, each of which has a *Sfi*I recognition sequence, JH97 (Sfi-HAS-forward primer) (SEQ ID NO. 11) and JH119 (Sfi-HAS-reverse primer) (SEQ ID NO. 12), respectively, pYHSA5 (Kang et al., J. Microbiol. Biotechnol., 1998, 8, 42) as a template, and Pfu polymerase (Stratagene, USA). PCR conditions included one cycle of 94□C for 5 min, and 25 cycles of 94□C for 30 sec, 55□C for 30 sec and 72□C for 2 min, followed by one final cycle of 72□C for 7 min. A PCR product of about 1.8 kb, which was an albumin gene, was obtained. Separately, the invertase gene was amplified by PCR using a set of primers, JH99 (Sfi-INV-forward) (SEQ ID NO. 13) and JH100 (SalI-INV-reverse primer) (SEQ ID NO. 14), and pRB58 as a template under the same conditions. The amplified invertase gene was

treated with *Sfi*I/*Sal*I, and was inserted along with the albumin gene treated with *Pst*I/*Sfi*I into *Pst*I/*Sal*I-digested pBluescript (Stratagene, USA). Then, pYHSA5 was digested with *Sac*I/*Pst*I to excise a fragment containing a *GAL* promoter and a portion of the albumin gene. This fragment, and a *Pst*I-*Sal*I insert excised from the plasmid prepared above, containing a portion of the albumin gene and the invertase gene, were co-ligated into the *Sac*I/*Sal*I sites of a YEG-HIR525 vector (Choi et al., Appl Microbiol Biotechnol., 1994, 42, 587), thereby generating pGHSA-INV2. A fusion expression vector of IL-2 and invertase was prepared as follows. The IL-2 gene was amplified by PCR using a set of primers, JH106 (Sfi-IL2-forward primer) (SEQ ID NO. 15) and JH107 (Sfi-IL2-reverse primer) (SEQ ID NO. 16), and pT7-hIL-2 (JK Jung, Korea Research Institute of Bioscience and Biotechnology) as a template. The amplified interleukin gene was inserted into the *Eco*RV site of pBluescript (Stratagene, USA), thus generating pBKS-IL2. The linearized form of the pBKS-IL2 through *Sfi*I digestion and a *Sac*I-*Sfi*I insert excised from the pGHSA-INV2, containing a *GAL* promoter and an *INV* secretory signal, were co-ligated into the *Sac*I/*Sfi*I sites of the pGHSA-INV2, thus generating pGIL2-INV2.

The pGHSA-INV2 vector expressing a fusion protein of albumin and invertase, the pGIL2-INV2 expressing a fusion protein of IL-2 and invertase, and the pRB58 expressing only invertase were individually transformed into a yeast strain (Y2805□*inv2*), which is deleted for its endogenous invertase gene and thus unable to grow in a sucrose medium. The transformed cells were smeared onto a medium (UD) containing glucose as a carbon source and a medium (YPSA) containing sucrose as a carbon source, and their growth was observed (FIG. 5). When cells were transformed with the pRB58 vector normally expressing invertase, they normally grew in both carbon sources. Also, when cells

were transformed with the pGHSA-INV2 vector in which invertase is fused to albumin leading to the high-level expression of the invertase, they grew well using both carbon sources. In contrast, when cells were transformed with the pGIL2-INV2 vector in which invertase is fused to IL-2 leading to the poor expression of the invertase, they grew normally on the glucose medium but rarely grew on the sucrose medium. This inability of the pGIL2-INV2-transformed cells to grow in the sucrose medium was believed to result from IL-2 being unable to be secreted from the cells and leads to block the secretion of invertase fused thereto. These results indicate that the use of an exogenous invertase gene, introduced into a yeast mutant (Y2805□inv2), which cannot grow on a sucrose medium due to deletion of its endogenous invertase gene, and secreted or not therein makes automatic screening of yeast cells possible.

EXAMPLE 3: Preparation of translational fusion partner screening vector using a non□producible protein human IL-2

In order to obtain suitable translational fusion partners capable of inducing secretion of a fusion protein using the pGIL2-INV2 vector in which IL-2 is fused to invertase, vectors having three reading frames for preparing a library, pYHTS-F0, F1 and F2, were prepared (FIG. 6).

PCR was carried out using sense primers having three reading frames and a *Bam*HI recognition site, JH120 (BamHI-IL2-1-forward primer) (SEQ ID NO. 17), JH121 (BamHI-IL2-2-forward primer) (SEQ ID NO. 18) and JH122 (BamHI-IL2-3-forward primer) (SEQ ID NO. 19), an antisense primer, JH123 (INV-1-reverse primer) (SEQ ID NO. 20), pGIL2-INV2 as a template, and Pfu polymerase (Stratagene, USA). PCR conditions included one cycle of 94□C for 3 min, and 25 cycles of 94□C for 30 sec, 55□C for 30 sec and 72□C for 1 min, followed by one final cycle of 72□C for 7 min. PCR products of about 1.2 kb, containing the IL-2 gene and a portion of the invertase gene, were obtained. Separately, PCR was carried out using a set of primers, JH124 (INV-forward primer) (SEQ ID NO. 21) and JH95 (INV-2-reverse primer) (SEQ ID NO. 22), and pGIL2-INV2 as a template under the same conditions, thus obtaining a fragment of about 0.9 kb containing a portion of the invertase gene. The

PCR products were purified from agarose gels. After each of the three 1.2-kb fragments having three reading frames and the 0.9-kb fragment were mixed, secondary PCR was carried out using sense primers, JH120 (SEQ ID NO. 17), JH121 (SEQ ID NO. 18), JH122 (SEQ ID NO. 19), and an antisense primer, JH95 (SEQ ID NO. 22). Three fragments of about 2.1 kb were obtained by agarose electrophoresis. The three recovered 2.1-kb fragments were digested with both *Bam*HI and *Sal*I and individually inserted into pGIL2-INV2 digested with both *Bam*HI and *Sal*I, thus generating pYHTS-F0, F1 and F2.

EXAMPLE 4: Preparation of suitable translational fusion partner library from yeast genome

A translational fusion partner library was prepared using chromosomal DNA from yeast *Saccharomyces cerevisiae* Y2805 (SK Rhee, Korea Research Institute of Bioscience and Biotechnology) and yeast *Hansenula polymorpha* DL-1 (ATCC26012). After each chromosomal DNA was partially digested with *Sau*3AI, DNA fragments ranging from 0.5 kb to 1.0 kb were purified from agarose gels, and ligated with a mixture of pYHTS-F0, F1 and F2 vectors digested with *Bam*HI and treated with calf intestine phosphatase (FIG. 6). Then, *E. coli* DH5□ was transformed with the ligated DNA, smeared onto ampicillin-containing LB medium (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl), and incubated at 37□ for one day. Using the library DNA prepared from the yeast chromosomal DNA, a library of about $5\square 10^4$ transformants was obtained. All transformants were recovered using sterile distilled water, and library DNA was isolated from the recovered transformants using a plasmid extraction kit (Bioneer, Korea).

EXAMPLE 5: Automatic screening of translational fusion partners suitable for a non-producible protein human IL-2

The library DNA prepared in Example 4 was transformed into yeast *Saccharomyces cerevisiae* Y2805Δ*gal1*Δ*inv2* (*Mat a ura3 inv2::Tc190 pep4::HIS3 gal1 can1)* using a lithium acetate procedure

(Hills et al., Nucleic Acids Res. 1991, 19, 5791). Then, the transformed cells were smeared onto UD minimal medium lacking uracil (0.67% yeast nitrogen base without amino acids, mixture of various amino acids of proper concentrations, 2% glucose), and YPGSA medium (1% yeast extract, 2% peptone, 2% sucrose, 0.3% galactose, 1 □g/ml antimycin A), and were incubated at 30□C for 5 days. The number of colonies that emerged on each of the media is given in Table 1, below, in which the number of transformants is compared before and after introduction of translational fusion partners. When yeast cells were transformed with only the vectors (pYHTS-F0, F1 and F2) used for library preparation, about 1□10^4 colonies were formed on the glucose medium, but the cells did not grow on the sucrose medium as expected. In contrast, when yeast cells were transformed with the yeast genome library, about 11 transformants grew on the sucrose medium, indicating that invertase is secreted with the aid of the introduced translational fusion partners.

TABLE 1

DNA introduced into Y2805Δ*gal1Δinv2* (host cells)	Number of transformants	
	UD minimal medium (glucose)	YPSGA (sucrose)
□		
pYHTS vectors	0	0
pYHTS+genomic library(*S. cerevisiae*)	~1×10^4	0
	~1×10^4	10
pYHTS+genomic library(*H. polymorpha*)	~1×10^4	1

EXAMPLE 6: Analysis of translational fusion partners

Transformants grown on the sucrose medium were cultured in YPD medium (1% yeast extract, 2% peptone, 2% glucose) for 24 hrs. After the cultured cells were harvested, they were lysed to isolate the introduced plasmids. The isolated plasmids were re-transformed into *E.*

coli. Plasmids were isolated again from the transformed *E. coli*, assessed for gene insertion by restriction mapping, and subjected to DNA sequencing analysis. As a result, the plasmids were found to contain four genes having different sequences, which served as translational fusion partners (Table 2: novel translational fusion partners inserted into plasmids isolated from transformants having grow in sucrose medium).

TABLE 2

Plasmids	Translational fusion partners	Yeast genes	Number of fused amino acids (total amino acid number)	Characteristics
pYHTS□TFP1	*TFP1*	*Yar066w*	105(203)	PRE, N□gly, Ser□rich, GPI
pYHTS□TFP2	*TFP2*	*Yar026c*	117(169)	PRE, N□gly
pYHTS□TFP3	*TFP3*	*Yjl158c*	104(227)	PRE□PRO,
pYHTS□TFP4	*TFP4*	Unknown	50(unknown)	O□gly, PIR PRE

6-1. Translational fusion partner 1 (TFP1)

The present inventors found translational fusion partner 1 (TFP1) (SEQ ID NO. 2) to be capable of effectively secreting a fusion protein of IL-2 and invertase into the extracellular environment. The TFP1 gene was identical to a yeast *S. cerevisiae* gene Yar066w. The Yar066w gene is similar to the -1,4-glucan-glucosidase (STA1) gene and encodes a protein containing a glycosyl-phosphatidylinositol (GPI) anchor. The Yar066w gene, which is of unknown function, has 70.3% and 72.7% sequence similarities to yeast *S. cerevisiae* genes of unknown function, Yol155c and Yil169c, respectively. In an amino acid sequence encoded by the Yar066w gene, the region fused to IL-2 consisted of 105 amino acid residues among the total number of 203 amino acid residues, and contained

a secretory signal of 23 amino acid residues for protein secretion, an N-glycosylation site and a serine/alanine-rich sequence.

6-2. Translational fusion partner 2 (TFP2)

The present inventors found translational fusion partner 2 (TFP2) (SEQ ID NO. 4) to be capable of effectively secreting a fusion protein of IL-2 and invertase into the extracellular environment. The TFP2 gene was identical to a yeast *S. cerevisiae* gene Yar026c. The Yar026c gene is of unknown function. In an amino acid sequence encoded by the Yar026c gene, the region fused to IL-2 consisted of 117 amino acid residues among the total number of 169 amino acid residues, and contained a secretory signal of 19 amino acid residues for protein secretion and three N-glycosylation sites.

6-3. Translational fusion partner 3 (TFP3)

The present inventors found translational fusion partner 3 (TFP3) (SEQ ID NO. 6) to be capable of effectively secreting a fusion protein of IL-2 and invertase into the extracellular environment. The TFP3 gene was identical to a yeast *S. cerevisiae* gene Yjl158c (PIR4/CIS3). The Yjl158c gene encodes an *O*-mannosylated protein covalently linked to the cell wall. The Yjl158c gene is known as a multicopy suppressor for a mutant deficient in the *Cik1* gene participating in cell division. In an amino acid sequence encoded by the Yjl158c gene, the region fused to IL-2 consisted of 104 amino acid residues among the total number of 227 amino acid residues, and contained a pre-secretory signal of 23 amino acid residues and a pro-secretory signal of 41 amino acid residues for protein secretion, a Kex2p cleavage site containing a sequence of Lys-Arg, and a PIR repeat sequence.

6-4. Translational fusion partner 4 (TFP4)

The present inventors found translational fusion partner 4 (TFP4) (SEQ ID NO. 8) to be capable of effectively secreting a fusion protein of IL-2 and invertase into the extracellular environment. The TFP4 gene is derived from *Hansenula polymorpha* and is of unknown function. The region fused to IL-2 consisted of 50 amino acid residues, which contained a

protein secretory signal of 18 amino acid residues.

EXAMPLE 7: Analysis of fusion proteins secreted into culture medium

To assess proteins secreted by yeast cells grown in a sucrose medium, yeast cells containing the four translational fusion partners described in Example 6 were cultured in YPDG medium (1% yeast extract, 2% peptone, 2% glucose, 0.3% galactose) for 40 hrs. After cells were removed, total proteins dissolved in the remaining culture supernatant were precipitated with acetone (final concentration: 40%) and analyzed by SDS-PAGE. However, each translational fusion partner did not appear as a single band because it had excessive glycosylation at a state of being fused to invertase. To solve this problem, invertase was removed from each vector (pYHTS-TFP1, 2, 3 and 4), and a translational termination codon was introduced into the IL-2 gene. In brief, to obtain a fragment including an IL-2 gene containing a *GAL* promoter, a TFP and a translational termination codon from each vector, PCR was carried out using a set of primers, JH132 (SacI-GAL-forward primer) (SEQ ID NO. 23) and JH137 (IL2-Term-reverse primer) (SEQ ID NO. 24). The amplified gene fragments were individually digested with *Sac*I/*Sal*I and inserted into a *Sac*I/*Sal*I-digested YEGα-HIR525 vector, thus generating pYIL-TFP1, 2, 3 and 4. The four IL-2 expression vectors were individually transformed into yeast cells. The resulting single colonies were cultured according to the same methods as described above, and the culture supernatants were analyzed by SDS-PAGE (FIG. 7). As shown in FIG. 7, strong protein bands having different sizes were found in the culture supernatants of yeast cells containing the IL-2 expression vectors except for pYIL-TFP2. These bands were confirmed by Western blotting using an anti-IL-2 antibody (FIG. 7), thus indicating that each secretion-inducing fusion protein is present at a state of being fused to IL-2. However, the size of the fusion proteins on SDS-PAGE was different from that predicted from the molecular weights of each translational fusion partner and the IL-2 gene. This difference was considered to be due to glycosylation of the fusion protein. Thus, each fusion protein was digested with Endo□H and analyzed by SDS-PAGE (FIG. 8). On an amino acid sequence, TFP1 was

found to have one consensus *N*-glycosylation sequence (amino acid residues 28-30), and TFP3 contained a consensus *O*-glycosylation sequence. TFP4 was found to have no glcosylation sequence. As predicted, in the case of the protein expressed by the pYIL-TFP1, a great decrease was found in molecular weight after Endo-H digestion, indicating that the protein expressed by the pYIL-TFP1 is *N*-glycosylated. In contrast, there was no change in molecular weight of the protein expressed by the pYIL-TFP3 upon Endo-H digestion because the protein is *O*-glycosylated. Also, there was no change in molecular weight of the protein expressed by the pYIL-TFP4.

EXAMPLE 8: Production of authentic proteins by Kex2p cleavage in cells

In order to produce the TFP-IL-2 fusion proteins, which were expressed by the vectors used in Example 7 and secreted into the medium, in the authentic form identical to native human IL-2, a cleavage site (Leu-Asp-Lys-Arg) recognized by Kex2p protease, which yeast cells themselves produce, was inserted between a TFP and IL-2 so as to automatically remove the TFP from cells. To introduce a Kex2p cleavage site into the pYIL-TFP1, PCR was carried out using the pYIL-TFP1 as a template with each of two sets of primers, JH132 (SEQ ID NO. 23) and HY22 (TFP1-LDKR-reverse primer) (SEQ ID NO. 25), and HY23 (TFP1-LDKR-forward primer) (SEQ ID NO. 26) and JH137 (SEQ ID NO. 24). A secondary PCR was carried out using as templates the amplified products, a fragment containing a *GAL* promoter and TFP1 and another fragment containing the IL-2 gene, which were electrophoresed and recovered from a gel, with a set of primers, JH132 (SEQ ID NO. 23) and JH137 (SEQ ID NO. 24). The secondarily amplified *GAL* promoter-TFP1-IL2 fragment was digested with *Sac*I/*Sal*I and inserted into a *Sac*I/*Sal*I-digested YEG-HIR525 vector, thus generating pYIL-KRTFP1. Also, to introduce a Kex2p cleavage site into the pYIL-TFP2, according to the same method as described above, PCR was carried out using the pYIL-TFP2 as a template with two sets of primers, JH132 (SEQ ID NO. 23) and HY20 (TFP2-LDKR-reverse primer) (SEQ ID NO. 27), and HY21 (TFP2-LDKR-forward primer) (SEQ ID NO. 28) and JH137 (SEQ ID NO. 24). A secondary PCR was carried out using two amplified gene fragments as

templates with a set of primers, JH132 (SEQ ID NO. 23) and JH137 (SEQ ID NO. 24). The secondarily amplified fragment was digested with *Sac*I/*Sal*I and inserted into a *Sac*I/*Sal*I-digested YEG-HIR525 vector, thus generating pYIL-KRTFP2. Further, to introduce a Kex2p cleavage site into the pYIL-TFP3, according to the same method as described above, PCR was carried out using the pYIL-TFP3 as a template with two sets of primers, JH132 (SEQ ID NO. 23) and HY17 (TFP3-LDKR-reverse primer) (SEQ ID NO. 38), and HY18 (TFP3-LDKR-Forward primer) (SEQ ID NO. 39) and JH137 (SEQ ID NO. 24). A secondary PCR was carried out using two amplified gene fragments as templates with a set of primers, JH132 (SEQ ID NO. 23) and JH137 (SEQ ID NO. 24). The secondarily amplified fragment was digested with *Sac*I/*Sal*I and inserted into a *Sac*I/*Sal*I-digested YEG-HIR525 vector, thus generating pYIL-KRTFP3. Yet further, to introduce a Kex2p cleavage site into the pYIL-TFP4, according to the same method as described above, PCR was carried out using the pYIL-TFP4 as a template with two sets of primers, JH132 (SEQ ID NO. 23) and HY24 (TFP4-LDKR-reverse primer) (SEQ ID NO. 29), and HY25 (TFP4-LDKR-forward primer) (SEQ ID NO. 30) and JH137 (SEQ ID NO. 24). A secondary PCR was carried out using two amplified gene fragments as templates with a set of primers, JH132 (SEQ ID NO. 23) and JH137 (SEQ ID NO. 24). The secondarily amplified fragment was digested with *Sac*I/*Sal*I and inserted into a *Sac*I/*Sal*I□digested YEG□□HIR525 vector, thus generating pYIL-KRTFP4.

Among the four vectors, pYIL-KRTFP1, pYIL-KRTFP3 and pYIL-KRTFP4 were individually introduced into a yeast 2805Δ*gal1*Δ*inv2* strain. Single colonies were picked and cultured in YPDG medium (1% yeast extract, 2% peptone, 2% glucose, 0.3% galactose) for 40 hrs. After cells were removed, the remaining culture supernatants were subjected to SDS-PAGE. As shown in FIG. 9, secreted proteins were found to have the same size as native human IL-2. Among the three TFPs inducing the secretory production of human IL2, TFP1 was found to be most effective in the secretory production of authentic IL-2.

The pYIL-KRTFP1, pYIL-KRTFP2, pYIL-KRTFP3 and pYIL-

KRTFP4 vectors were deposited at an international depository authority, KCTC (Korean Collection for Type Cultures; 52, Oun-dong, Yusong-ku, Taejon, Korea) on November 11, 2003, and assigned accession numbers KCTC 10544BP, 10545BP, 10546BP and 10546BP, respectively.

EXAMPLE 9: Analysis of characteristics of the translational fusion partner 1 (TFP1)

The TFP1, identified as a translational fusion partner most effectively inducing the secretory production of IL-2, was assessed to determine whether any of specific sequences present on the TFP1 sequence, a secretory signal (a), an *N*-glycosylation site (b), a serine/alanine-rich sequence (c), an additional sequence (d) and a 5-UTR (5-untranslated region) (e), directly affects the secretion of the non□producible protein IL-2. To do this, as shown in FIG. 10, deletion mutants of the TFP1 gene, in which each specific sequence was deleted, were prepared. First, to remove the additional sequence (d) having no unique property from the TFP1 sequence, PCR was carried out using the pYIL-KRTFP1 as a template with a set of primers, JH143 (XbaI-TFP1-d-reverse primer) (SEQ ID NO. 31) and JH132 (SEQ ID NO. 23). The amplified DNA fragment contained the TFP1-1, which was deleted in the *GAL* promoter and the (d) sequence of the TFP1 sequence. To remove the additional sequence (d) and the serine/alanine-rich sequence (c) from the TFP1 sequence, PCR was carried out using the pYIL-KRTFP1 as a template with a set of primers, JH142 (XbaI-TFP1-c-reverse primer) (SEQ ID NO. 32) and JH132 (SEQ ID NO. 23). The amplified fragment contained the TFP1-2, which was deleted in the *GAL* promoter, the (c) sequence and the (d) sequence. Also, to remove the (d) sequence, the (c) sequence and the *N*-glycosylation site (b) from the TFP1 sequence, PCR was carried out using the pYIL-KRTFP1 as a template with a set of primers, JH141 (XbaI-TFP1-b-reverse primer) (SEQ ID NO. 33) and JH132 (SEQ ID NO. 23). The amplified fragment contained the TFP1-3, which was deleted in the *GAL* promoter and the sequences (c), (d) and (b). To introduce a Kex2p cleavage site into the IL-2 gene, PCR was carried out using the pYIL-KRTFP1 as a template with a set of primers, JH140 (SpeI-XbaI-LDKR-forward primer) (SEQ ID NO. 34) and JH137 (SEQ ID NO. 24). The amplified IL-2 fragment was

purified, digested with *Spe*I and *Sal*I, and, along with each of the three obtained fragments (TFP1-1, 2 and 3) digested with *Sac*I and *Xba*I, inserted into a YEG-HIR525 vector predigested with *Sac*I and *Sal*I, thus generating, as shown in FIG. 10, pYIL-KRT1-1, pYIL-KRT1-2 and pYIL-KRT1-3, respectively. To remove the 5'-UTR of the TFP1, PCR was carried out using the pYIL-KRTFP1 as a template with a set of primers, HY38 (TFP1-UTR-forward primer) (SEQ ID NO. 35) and JH137 (SEQ ID NO. 24). The amplified gene was purified, digested with *Bam*HI/*Sal*I, and ligated along with a *Sac*I/*Bam*HI-digested *GAL10* promoter into a *Sac*I/*Sal*I-digested YEG-HIR525 vector, thus generating pYIL-KRT1-4 (FIG. 10).

The four plasmids, pYIL-KRT1-1, pYIL-KRT1-2, pYIL-KRT1-3 and pYIL-KRT1-4, were transformed into yeast cells. Single colonies were cultured, and culture supernatants were subjected to SDS-PAGE. As shown in FIG. 11, an IL-2 band was found only in a culture supernatant of cells transformed with the pYIL-KRT1-3 containing all of the secretory sequence, the *N*-glycosylation site and the serine/alanine-rich sequence. The IL-2 band was not observed in culture supernatants of cells transformed with the pYIL-KRT1-2 deleted for the serine/alanine-rich sequence and cells transformed with the pYIL-KRT1-1 deleted in both the serine/alanine-rich sequence and the *N*-glycosylation site. These results indicate that three characteristic sequences (the secretory sequence, the *N*-glycosylation site and the serine/alanine-rich sequence) present in the TFP1 are required for effectively inducing IL-2 secretion. Also, when the TFP1 was deleted in its 5'-UTR, protein expression levels increased more than about three times.

The pYIL-KRT1-3 and pYIL-KRT1-4 vectors were deposited at an international depository authority, KCTC (Korean Collection for Type Cultures; 52, Oun-dong, Yusong-ku, Taejon, Korea) on November 11, 2003, and assigned accession numbers KCTC 10548BP and 10549BP, respectively.

EXAMPLE 10: Secretion and production of human G-CSFC using the translational fusion partners TFP-1

In order to determine whether the translational fusion partner (TFP-1) obtained using the non-producible protein human IL-2 are effective in the secretion of other non-producible human proteins, a non-producible protein, human G-CSF was fused to TFP-1 and was expressed in yeast cells and assessed for its secretion. The human G-CSF gene was obtained as follows. PCR was carried out using a human cDNA library with a set of primers, JH144 (GCSF-forward primer) (SEQ ID NO. 36) and JH145 (GCSF-reverse primer) (SEQ ID NO. 37). The amplified gene was digested with *Xba*I/*Sal*I and inserted into the *Xba*I/*Sal*I sites of pYIL-KRTFP1, thus generating pYGCSF-TFP1. A secretory signal MFα derived from a mating factor alpha, which is very frequently used in yeasts for secretion and production of proteins was used as a control. pYGCSFα-MFα was prepared by inserting G-SCF genes treated with *Xba*I/*Sal*I into X*ba*I/*Sal*I part of YEGα-HIR525.

To express human G-CSF in yeast cells, the pYGCSF-TFP1 and pYGCSF-MFα were transformed into yeast cells. Single colonies were isolated and cultured, the culture supernatants were subjected to SDS-PAGE and Western blotting using an anti-G-CSF antibody. The results are given in FIG. 12. In case of MFα used as a control, G-CSF band was not confirmed, but in case of TFP-1, G-CSF band was confirmed. Western blotting with an anti-G-CSF antibody (Chemicon, USA) demonstrated that TPF3 is most effective in the secretory production of G-CSF. Thus, because TFP-1 was demonstrated to exert maximal secretion efficiency according to the type of protein, the TFP-1 of the present invention was considered to be very useful as translational fusion partners capable of secreting various non-producible proteins other than IL-2 and G-CSF.

[Advantageous Effects]

Rapid screening of suitable translational fusion partners (TFPs)

capable of inducing expression or secretory production of non-producible proteins, which are difficult to produce using conventional recombinant production methods, from a variety of genetic sources can be realized.

The present invention allows the cost-effective large-scale production of various proteins that are not able to recombinantly produce or are expressed in low levels through the rapid screening and use of suitable TFPs.

CLAIMS

1. A method of screening a suitable translational fusion partner (TFP) for producing a non-producible protein, comprising:

(1) preparing an automatic screening vector including a fusion gene (X-R) in which a gene (X) encoding a non-producible target protein is linked in frame to a reporter gene (R) for automatic screening;

(2) linking a gene library including a TFP inducing secretion of the non-producible fusion protein (X-R) to the automatic screening vector to create a TFP library;

(3) transforming cells having no activity of the reporter gene with the TFP library to detect the activity of a reporter protein; and

(4) isolating a gene from transformed cells exerting the activity of the reporter protein and analyzing properties of the TFP.

2. The method according to claim 1, wherein the reporter gene is a gene selected from among invertase, amylase, glucoamylase, galactosidase, sucrase, cellulase, xylanase and maltase.

3. The method according to claim 1, wherein the non-producible protein is selected from among cytokines, serum proteins,

immunoglobulins, interferons, colony stimulating factors, stem cell factor (SCF), phospholipase A2-activating protein (PLAP), insulin, tumor necrosis factor (TNF), growth factors, lactoferrin, hormones, calcitonin, calcitonin gene related peptide (CGPR), enkephalin, somatomedin, erythropoietin, hypothalamic releasing factor, prolactin, chorionic gonadotropin, tissue plasminogen activator, growth hormone releasing peptide (GHPR), thymic humoral factor (THF), anticancer or antibiotic peptides, carbohydrate-specific enzymes, proteolytic enzymes, lipases, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

4. The method according to claim 1, wherein a non-producible protein is a human interleukin-2(IL-2) or a human G-SCF.

5. The method according to claim 1, wherein the genomic DNA or cDNA is derived from among *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Yarrowia*, *Saccharomyces*, *Aspergillus*, *Penicillium*, *Rhizopus* and *Trichoderma*.

6. The method according to claim 1, wherein the cells having no activity of the reporter gene are selected from among *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Yarrowia*, *Saccharomyces*, *Aspergillus*, *Penicillium*, *Rhizopus* and *Trichoderma*.

7. The method according to claim 1, wherein the screening vector comprises a promoter gene, a gene encoding a target protein, which is deleted for translation initiation and termination codons, and a reporter gene fused in frame to the gene encoding the target protein.

8. The method according to claim 1, wherein the promoter contained in the screening vector is selected from among *GAPDH, PGK, ADH, PHO5, GAL1* and *GAL10*.

9. A method of screening a translational fusion partner, comprising:

(1) preparing a yeast mutant strain deleted for its endogenous invertase gene INV2(I);

(2) preparing yeast high-throughput selection (HTS) vectors, pYHTS vectors (pYHTS-F0, pYHTS-F1 or pYHTS-F2) containing a gene (X-I) in which an invertase gene (I) is fused in frame to a non-producible protein gene (X) and which is controlled in expression under a yeast GAL10 promoter;

(3) preparing a translational fusion partner library from yeast genes capable of secreting the fusion gene (X-I) of an invertase and a

non-producible protein in the pYHTS vectors;

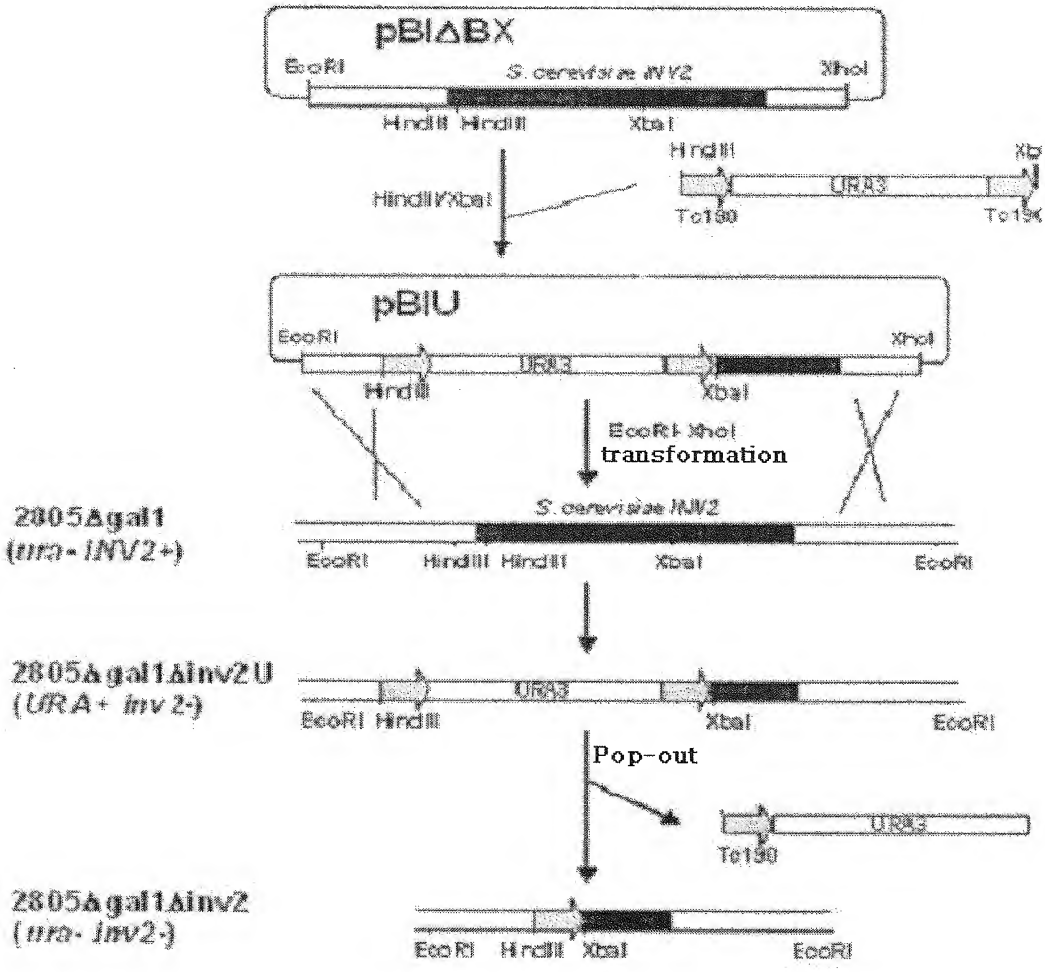
(4) transforming the library into the yeast mutant strain prepared at step (1) and performing automatic screening on a medium containing only sucrose as a carbon source;

(5) detecting a protein secreted into the medium by culturing yeast cells grown on the sucrose medium; and

(6) isolating genes from the yeast cells and analyzing properties of the translational fusion partner.

Figure

FIG. 1


pBIΔBX
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S. cerevisiae INV2
XhoI
HindIII HindIII
XbaI
HindIII/XbaI
HindIII
URA3
pBIU
EcoRI
XhoI
HindIII
XbaI
EcoRI XhoI
transformation
2805Δgal1
(ura- INV2+)
S. cerevisiae INV2
EcoRI
HindIII HindIII
XbaI
EcoRI
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URA3
EcoRI HindIII
XbaI
EcoRI
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URA3
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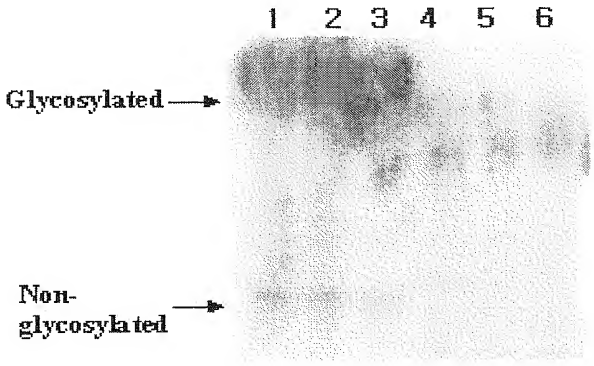
XbaI
EcoRI

FIG. 2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Glycosylated
Non-
glycosylated

FIG. 3

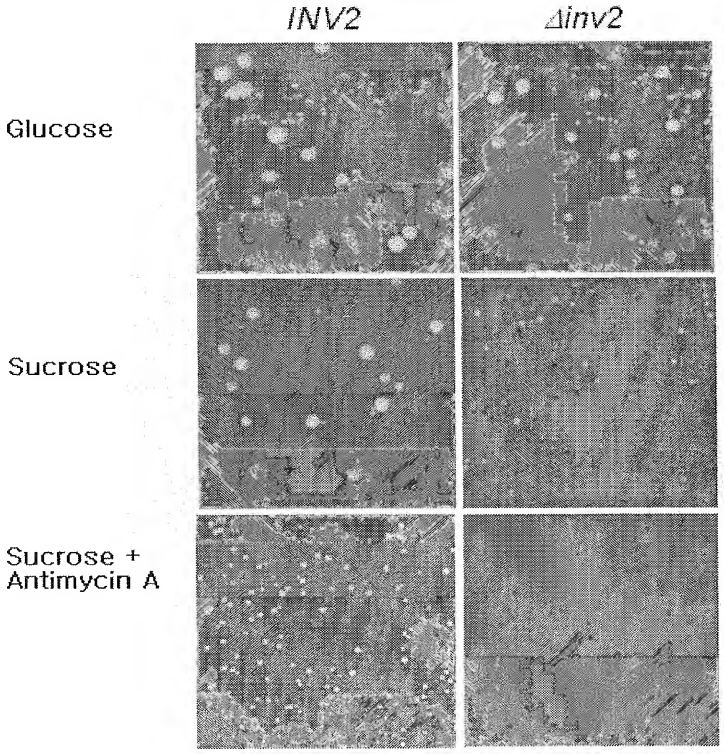

INV2
Δinv2
Glucose
Sucrose
Sucrose +
Antimycin A

FIG. 4

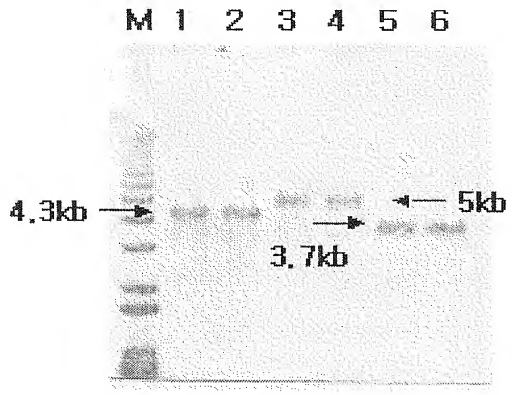


FIG. 5

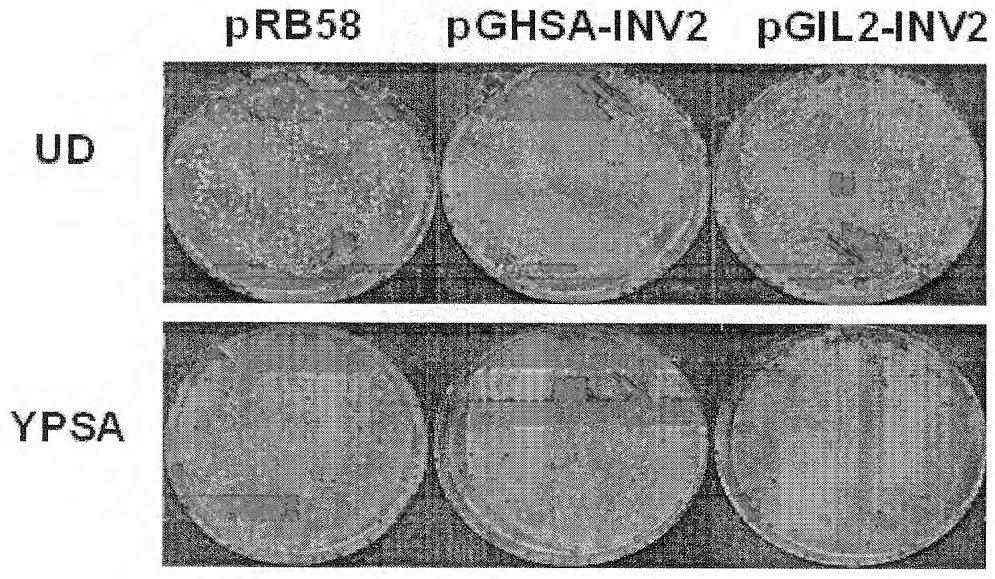
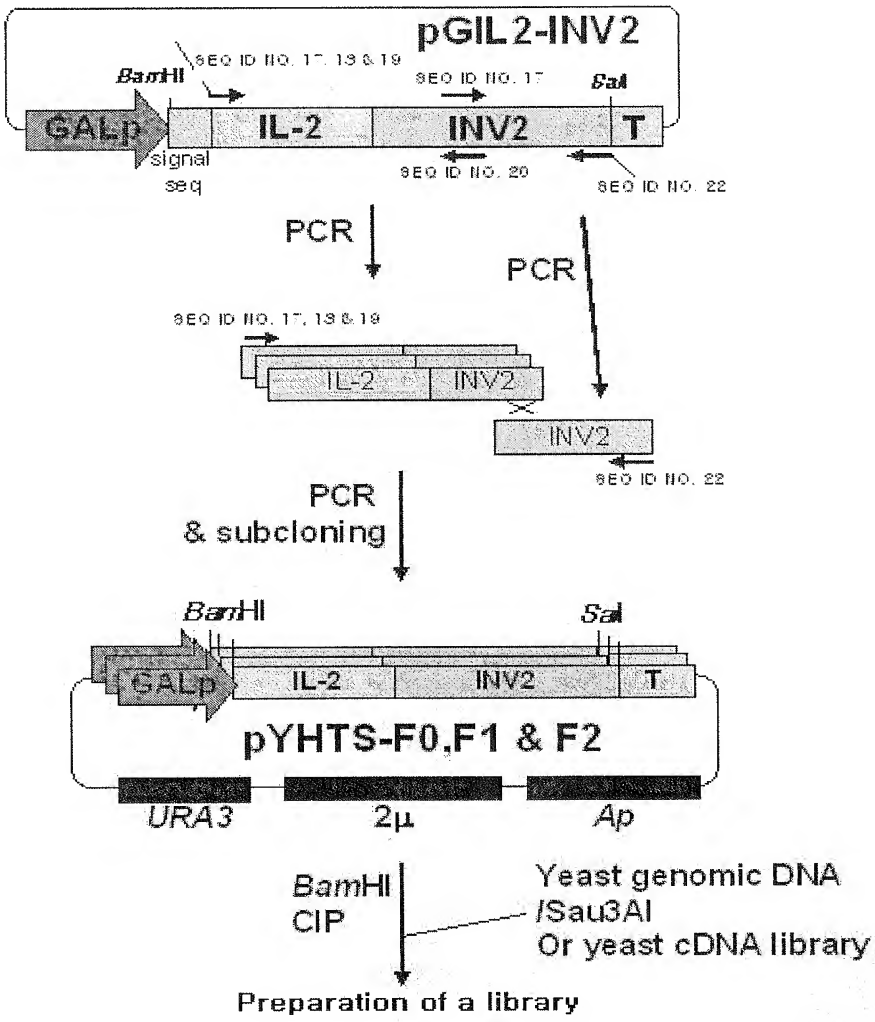


FIG. 6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SEQ ID NO. 17
SalI
GALp
IL-2
INV2
T
signal seq
SEQ ID NO. 20
SEQ ID NO. 22
PCR
PCR
SEQ ID NO. 17, 18 & 19
IL-2
INV2
INV2
SEQ ID NO. 22
PCR
& subcloning
BamHI
SalI
GALp
IL-2
INV2
T
pYHTS-F0,F1 & F2
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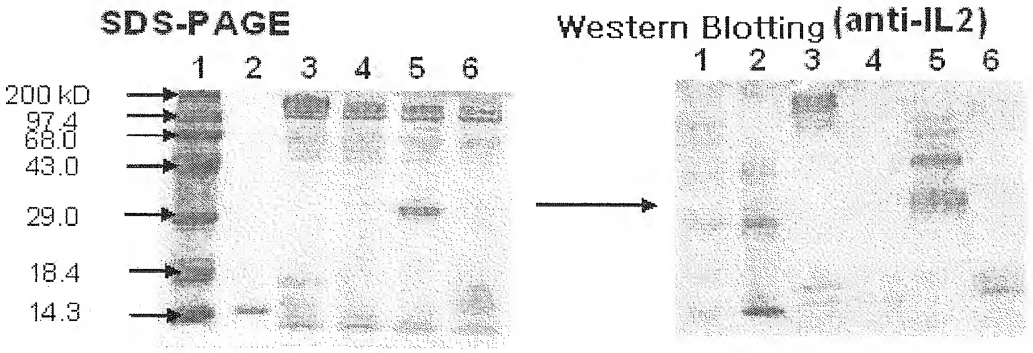
2μ
Ap
BamHI
CIP
Yeast genomic DNA
/Sau3AI
Or yeast cDNA library
Preparation of a library

FIG. 7


SDS-PAGE
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43.0
29.0
18.4
14.3
Western Blotting (anti-IL2)
1 2 3 4 5 6

FIG. 8

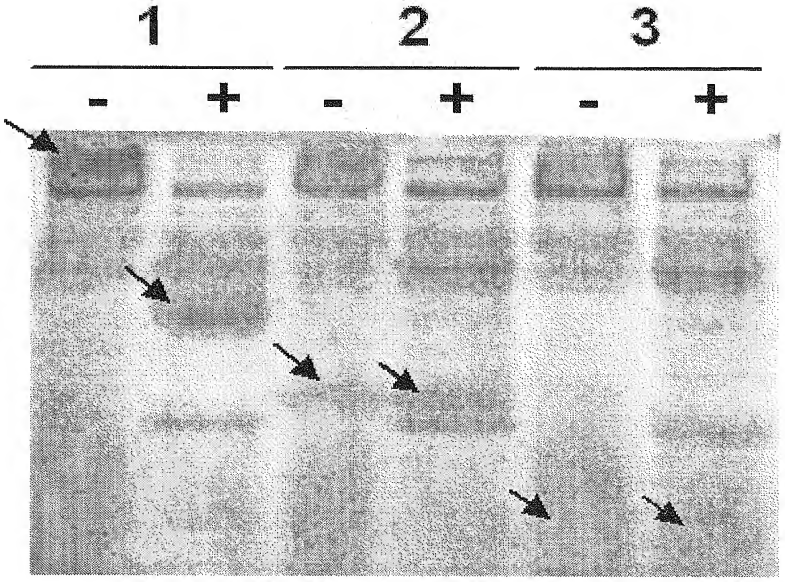

1
2
3
- + - + - +

FIG. 9

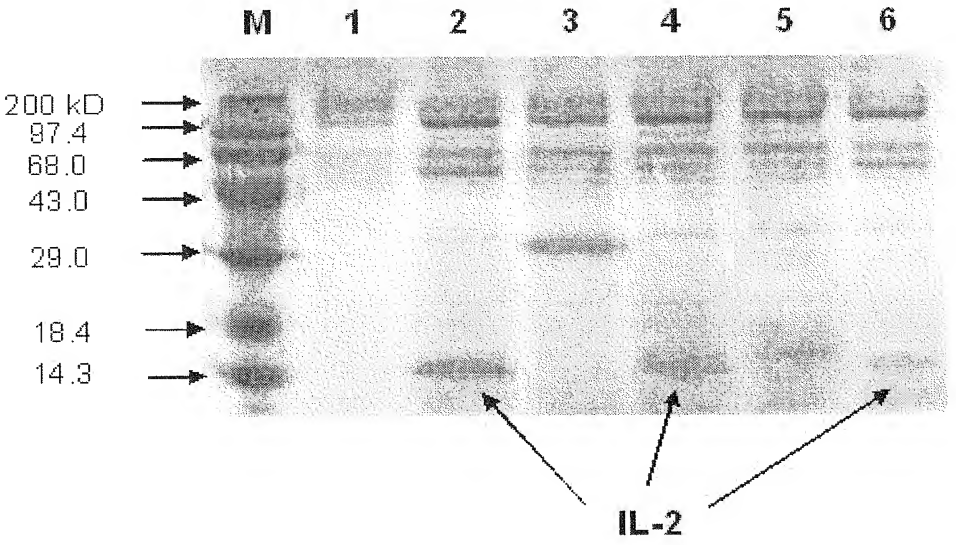
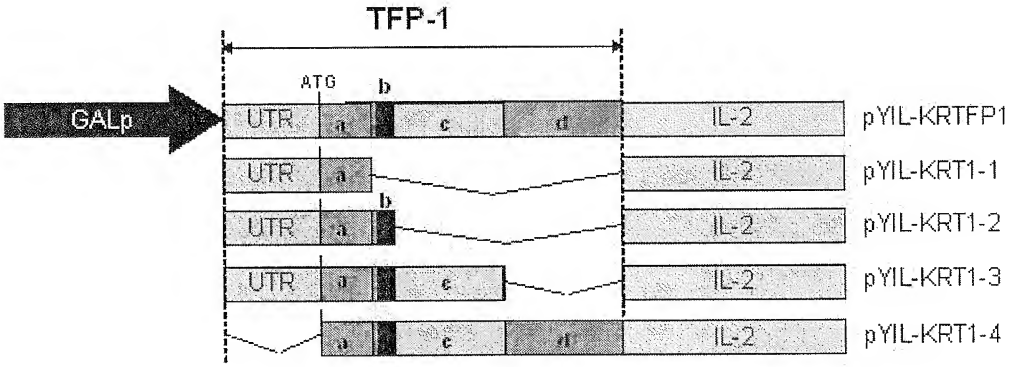
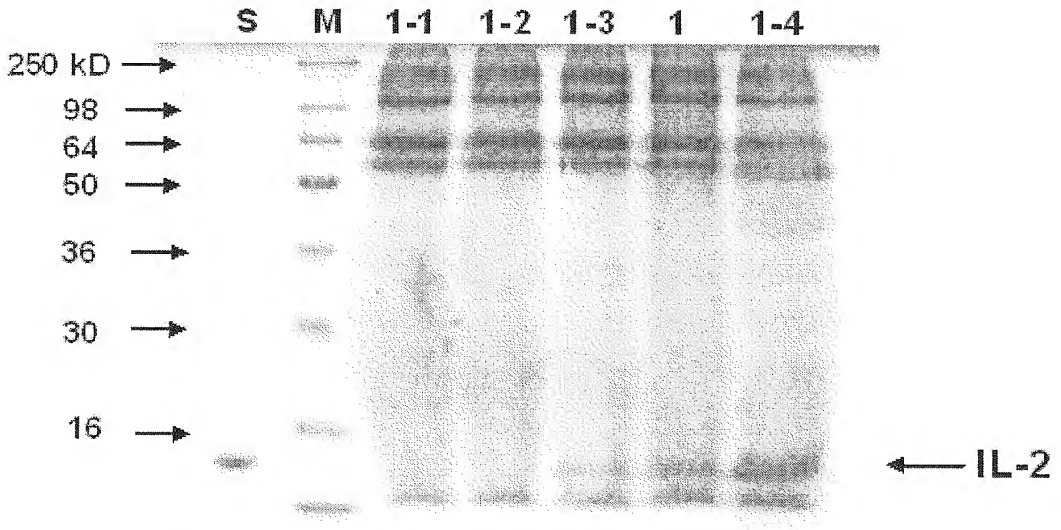


FIG. 10



a: Signal sequence
b: N-glycosylation site
c: Ser, Ala-rich sequence
d: Additional sequence
UTR: 5'-untranslated region

FIG. 11


S
M
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1-3
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1-4
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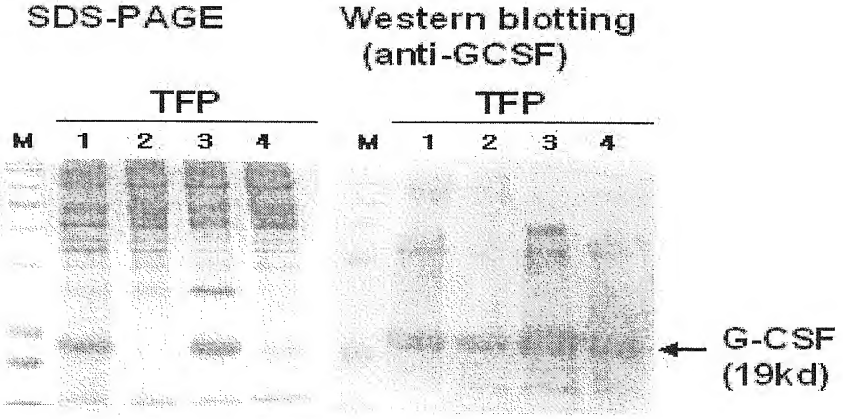
98
64
50
36
30
16
IL-2

FIG. 12


SDS-PAGE
Western blotting
(anti-GCSF)
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TFP
M
1
2
3
4
M
1
2
3
4
G-CSF
(19kd)

Sequence Listing

<110> Korea Research Institute of Bioscience and Biotechnology

<120> Rapid screening method of suitable translational fusion partners for producing recombinant proteins

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35 40 45

Thr Ala Ser Asp Ser Leu Ser Ser Ser Asp Gly Thr Val Tyr Leu Pro
50 55 60

Ser Thr Thr Ile Ser Gly Asp Leu Thr Val Thr Gly Lys Val Ile Ala
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100 105

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aatcgtttta acaaattcca agctgctgtc gctttggccc tactctctcg cggcgctctc 180

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tcagctagtg caagtgccac cgcttccgac tcactttctt ccagtgacgg taccgtttat 300

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35 40 45

Ala Leu Leu Ser Ile Pro Trp Asn Gly Pro Pro Glu Ser Leu Arg Asp
50 55 60

Ile Asn Leu Ile Glu Leu Glu Pro Gln Val Ala Leu Tyr Leu Leu Glu
65 70 75 80

Asn Tyr Ile Asn His Tyr Tyr Asn Thr Thr Arg Asp Asn Lys Cys Pro
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Arg Ser Leu Asn Asp
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aaaaatggag acggtactaa tgaacagtat gcaactgcac tactttctat tccctggaat 240

ggacctcctg agtcattgag ggatattaat cttattgaac tcgaaccgca agttgcactc 300

tatttgctcg aaaattatat taaccattac tacaacacca caagagacaa taagtgccct 360

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gatc 424

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5 9 14 19

Ala Thr Ala Ser Ala Glu Gly Tyr Thr Pro Gly Glu Pro Trp Ser Thr
24 29 34

Leu Thr Pro Thr Gly Ser Ile Ser Cys Gly Ala Ala Glu Tyr Thr Thr
39 44 49

Thr Phe Gly Ile Ala Val Gln Ala Ile Thr Ser Ser Lys Ala Lys Arg
54 59 64

Asp Val Ile Ser Gln Ile Gly Asp Gly Gln Val Gln Ala Thr Ser Ala
69 74 79 84

Ala Thr Ala Gln Ala Thr Asp Ser Gln Ala Gln Ala Thr Thr Thr Ala
89 94 99

Thr Pro Thr Ser Ser Glu Lys Ile
104

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acgtccacaa acaaaaacaa aaataagaaa taggttgaca gtgggtgaaa aattctcgaa 180

ggtttcatct ccaaacagtc agtatataag tattcgggaa agagagccaa tctatcttgt 240

ggtgggtcta tcttaacctt ctcttttgg cagtagtaat tgtaaatcaa gacacataaa 300

actatttcac tcgctaaact tacatctaaa atgcaattca aaaacgtcgc cctagctgcc 360

tccgttgctg ctctatccgc cactgcttct gctgaaggtt acactccagg tgaaccatgg 420

tccaccttaa ccccaaccgg ctccatctct tgtggtgctg ccgaatacac taccaccttt 480

ggtattgctg ttcaagctat tacctcttca aaagctaaga gagacgttat ctctcaaatt 540

ggtgacggtc aagtccaagc cacttctgct gctactgctc aagccaccga tagtcaagcc 600

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<211> 50
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<213> Hansenula polymorpha

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5 9 14 19

Met Ala Ala Pro Val Glu Ser Leu Ala Gly Thr Gln Arg Tyr Leu Val
24 29 34

Gln Met Lys Glu Arg Phe Thr Thr Glu Lys Leu Cys Ala Leu Asp Asp
39 44 49

Lys Ile
54

<210> 8
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<212> DNA
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ccacgttagg cgggggggatg gctgcaccgg ttgagtctct ggccgggacc caacggtatc 120

tggtgcaaat gaaggagcgg ttcaccacag agaagctgtg tgctttggac gacaagatc 179

<210> 9
<211> 71
<212> PRT
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<400> 9
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1 5 10 15

Leu Ser Arg Gly Ala Leu Gly Asp Ser Tyr Thr Asn Ser Thr Ser Ser
20 25 30

Ala Asp Leu Ser Ser Ile Thr Ser Val Ser Ser Ala Ser Ala Ser Ala
35 40 45

Thr Ala Ser Asp Ser Leu Ser Ser Ser Asp Gly Thr Val Tyr Leu Pro
50 55 60

Ser Thr Thr Ile Ser Gly Asp
65 70

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acttccgtct cgtcagctag tgcaagtgcc accgcttccg actcactttc ttccagtgac 180

ggtaccgttt atttgccatc cacaacaatt agcggtgatc tcacagttac tggtaaagta 240

attgcaaccg aggccgtgga agtcgctgcc ggtggtaagt tgactttact tgacggtgaa 300

aaatacgtct tctcatctga tcctctaga 329

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<210> 15
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<210> 17
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<400> 17
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<210> 18
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<223> JH121 primer

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<210> 24
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<210> 26
<211> 46
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<210> 28

<211> 46
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<210> 30
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<223> HY25 primer

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<210> 31
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<223> JH142 primer

<400> 32
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<210> 33
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<210> 34

<211> 25

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<223> JH140 primer

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<211> 42

<212> DNA

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<223> HY38 primer

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<223> HY17 primer

<400> 38
gaacttgaag taggtgccct tttatcaagg atcttttcgg agc 43

<210> 39
<211> 43

<212> DNA

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<220>

<223> HY18 primer

<400> 39

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